Chronic Exposure to Biomass Ambient Particulate Matter Triggers Ams Polarization and Activation in Rat Lung

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Research

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Abstract

Background: Chronic Obstructive Pulmonary Disease (COPD) is a chronic inflammatory disease in which a variety of immune cells are involved; among them, the role of alveolar macrophages (AMs) remains unknown in the pathogenesis and development of COPD. We aimed to study the function of AMs from different stages of chronic biomass fuel (BMF) exposed rats, and investigate the signal pathways which regulate AMs polarization.

Methods: 180 male Sprague-Dawley rats were divided into BMF group and clean air control (CON) group. After BMF exposure for 4 days, 1 month, and 6 months, the cytokine secretion and function of AMs were determined by flow cytometry, and further confirmed in mRNA level by qPCR, in protein level by WB and immunofluorescent assay. Bone marrow derived macrophages(BMDMs) were cultured, and PPAR γ agonist, PPAR γ KO lentivirus, and TGF β 1 were used as the intervention in vitro.

Results: We found that pro-inflammatory factors increased, while CD206 in AMs decreased remarkably at 4 days. Interestingly, pro-inflammatory macrophages shared a feature of anti-inflammatory macrophages. Consistent with IL4 upregulated in BALF, p-Stat6 and PPAR γ in AMs elevated at 4 days. After BMF exposure for 6 months, CD206, TGF β 1, and p-Smad3 were significantly higher than the control groups. PPAR γ reversed the M1 phenotype induced by PM, and drove the macrophages into the M2 phenotype in vitro.

Conclusion: We demonstrated that the dynamic phenotype and functional change of AMs during BMF exposure, and both PPAR γ and TGF β 1 were important molecules regulated AMs' function.

Background

Chronic Obstructive Pulmonary Disease (COPD) seriously endangers human health, with a high disability rate and fatality rate, causing a huge economic and social burden. According to an epidemiological study in China, the prevalence of COPD among people over 40 years old has increased to 13.7%¹. Factors that influence COPD development and progression are extremely complex, and smoking is not the only factor. Almost 3 billion people worldwide use biomass wood as the main source of energy for cooking, heating, and other household needs. Indoor biomass smoke exposure increases the risk of COPD. Moreover, a high level of biomass smoke exposure accounts for 50% of deaths in patients with COPD in developing countries², and is associated with the increase of COPD hospitalization³⁻⁷. Improvements in cooking fuels and kitchen ventilation had efficient effects on the decline in FEV_{1.0}⁸. Although the pathologic features of COPD patients induced by biomass ambient particulate matter are less severe than those of smoking COPD patients⁹, its danger should not be ignored. However, the molecular pathway of pathological damage caused by biomass ambient particulate matter in vivo and vitro remains less understood.

COPD is characteristic of a mixture of small airway disease and parenchymal destruction. Most research focused on airway inflammation. Recently studies have linked changes to host defense and lung immunity. Alveolar macrophages form the first line of immune defense in lung tissue and play an important role in maintaining lung local immune homeostasis. They contact outside air constantly, and perform their pattern recognition receptors to recognize invading pathogens and initiate the inflammatory response. When the dangerous substances are cleared, macrophages can secrete anti-inflammatory mediators and growth factors to promote the elimination of inflammation and tissue repair. Immune phenotype and function of AMs are greatly affected by the local microenvironment of the alveolar lumen.

Macrophages can be classified into classical activated M1 macrophage and alternative activated M2 macrophage¹⁰. When stimulated by Th1 cytokines such as interferon γ (IFN- γ) and toll-like receptor signaling, M1 activates¹¹. M1 expresses CD86, secretes IL1 and TNF α , and has a strong ability to kill pathogenic microorganisms¹². M1 secretes IL6, IL12, and IL23 to promote the differentiation of Th1 and Th17 cells and promote the inflammatory response¹³. The signal pathway molecules include Stat1, NF κ B, and mitogen-activated protein kinases(MAPKs). The antigen presentation capacity of AMs is very weak, which helps the macrophages not respond to harmless pathogens and reduce the release of inflammatory cytokines^{14,15}. When stimulated by Th2 cytokines such as IL4 and IL13, M2 activates and expresses CD206, CD163, TGF β , tyrosine-protein kinase, arginase 1, Stat6, and Stat3, which are involved in tissue repair and anti-inflammatory effects^{13,16,17}. M2 also secretes EGF and VEGF growth factors to promote tissue repair¹⁷. M2 is divided into several subgroups. The traditional M2 phenotype was known as M2a, and macrophages stimulated with IL10 were called M2c. M2a plays a critical role in attenuating inflammation and tissue repair, while M2c promotes tissue remodeling. With stimulation by IL4, nuclear receptor PPAR γ in macrophages is upregulated, skewing macrophage towards M2a phenotype^{18–20}. M2-derived TGF β contributes to fibrosis pathology by upregulation of α -smooth muscle actin(α -SMA) via the Smad pathway.

AMs increased in COPD patients, and released a number of inflammatory mediators to contribute to pathology^{21,22}. In contrast, it's reported that AMs in BALF of COPD patients were the high expression of M2 markers²³. But Bazzan E and co-workers have shown that both iNOS and CD206 were expressed by macrophages in the lungs of non-COPD smokers and COPD patients, indicated that macrophages in the lungs were polarized bidirectionally²⁴. The function of AMs in COPD is controversial. Diversity and plasticity are characters of macrophages in vivo²⁵. In order to maintain the homeostasis of the body, immunity is always in a dynamic change during the occurrence and development of the disease, and so do AMs. In the lung of animal models exposed to nitrogen mustard, M1 macrophages were predominant at 1–3 days, while M2 macrophages appeared at 28 days²⁶. Another study also showed M1 and M2 macrophages were activated sequentially in the lung after exposure of carbon nanotubes, which caused pulmonary injury progressing to fibrosis²⁷. But there is no research about the dynamic change of AMs' immune function in pathogenesis and development of COPD.

In this study, by establishing the biomass ambient particulate matter exposed rat model, we showed phenotypic change and immune function of AMs in vivo. To gain a better understanding how alveolar macrophage switched in COPD progress, we also investigated the dynamic change and function of some transcriptional factors for AMs activation.

Methods

Animals. 180 male Sprague-Dawley rats (170-200g, 6-8weeks old) were housed in the laboratory animal center of Guangzhou Medical University in barrier condition. The rats were randomly divided into biomass fuel (BMF) group and clean air control (CON) group. The experimental protocol and animal care was in compliance with the guiding principles for the care and use of laboratory animals recommended by the Chinese Association for Laboratory Animal Science Policy. Guangzhou Medical University Animal Research Ethics Board approved all experiments. The animal facility maintained a 12 hours light/dark cycle, and room temperature was fixed at $20 \pm 2^{\circ}\text{C}$ with 45–65% relative humidity.

BMF Exposure System. Rats were exposed to smoke produced by smoldering China fir saw-dust (2g/per heating panel/per time) for two 2-hours periods, 5 days per week, for 4 days, 1 month and 6 months. The BMF exposure system primarily consisted of a wood-burning unit and a whole body exposure unit. The size of the animal exposure chamber was 265×205×140mm(L×W×H). The BMF was generated by eight heating panels (500w), which were connected in series in wood-burning chamber. Each heating panel had worked for 20 minutes, then the next heating panel started. Biomass fuel smoke was set into the animal exposure chamber. Besides, there were two sampling ports to monitor various characteristics of exposure PM and gas in exposure chamber.

PM collection and extraction. PM was collected from the burning of China fir during high-temperature combustion with moderate air supply(April 23-May 6, 2015) in accordance with the procedures described previously²⁸. High volume sampler (TE-6070, Tisch, USA) equipped with a PM2.5 selective-inlet head($1.13\text{m}^3/\text{min}$) was used to collected particle. PM was collected on the fiber membrane filters with $1.6\mu\text{m}$ pore size for up to 2 hours. Exposed filters were soaked in water for 10 min and then in dichloromethane for 4 hours. The extracted solution was lyophilized and mixed. The weight of PM was defined as increase amount for each filter. The PM sample was dissolved in DMSO to 100mg/ml, and then diluted with culture medium to provide a concentration of less than 0.01% DMSO.

Sample preparation and isolation of AMs. Rats were sacrificed after 4 days, 1 month, and 6 months exposure period. Bronchoalveolar lavage fluid (BALF) was conducted by instilling the lungs sequentially with 8ml ice cold PBS for 4 times. BALF was centrifuged to obtain the cells and supernatants. The cells were suspended with 1ml PBS and counted with a cell counter (Millipore Scepter2.0, USA).

Bone marrow-derived macrophages culture and stimulation. Bone marrow cells were obtained from the femur and tibia bones, and were incubated in RPMI-1640 medium supplemented with 10% heated-inactivated fetal bovine serum and recombinant rat GM-CSF (10ng/ml, Peprotech) at 10ng/ml for 7days

as described²⁹. PM was used at a concentration ranging from 0 to 45µg/ml. Rosiglitazone (1µM, Sigma), IL4(50ng/ml, Peprotech) and TGFβ1(10 ng/ml, Peprotech) were added 1 hour before PM treatment.

CRISPR/Cas9 mediated PPAR γ gene knockout in BMDMs. LentiCrisp/Cas9 system was used to knockout PPAR γ genes in BMDMs using GeCKO LentiCrisp Resource Tool. Guide RNAs(gRNA) were designed using MIT's online webpage(<http://crispr.mit.edu/>).

The gRNA sequences for PPAR γ were: 5'CCTGTGGAGGTCCCATAATA3' and 5'TAATACCTTACCTAGTATCG3'. The vector included Cas9 gene. Lentivirus to knockout PPAR γ was constructed by Cyagen Biosciences(Guangzhou, China).

Gene expression. Total RNA was extracted and reverse transcribed by using PrimeScript RT reagent Kit with gDNA Eraser (Takara). Quantitative real-time PCR (qPCR) was performed by using TB Green Premix Ex Taq (Takara). The reactions were run in CFX Real-Time Detection System (BIO-RAD). The primer sequences for quantitative real-time PCR exhibited in Supplementary Table 1.

Flow cytometry. AMs were incubated with Fixable Viability Dye eFluor450 (Thermo Scientific) for 30 minutes at 4°C. After blocked with Fc receptors (BD Pharmingen), cells were incubated with CD206-PE (Polyclonal, IgG, R&D Systems), PE Isotype Control (IgG, R&D Systems), CD86-PE (clone 24F, IgG1,κ, BD Pharmingen), and PE Isotype Control (clone 24F, IgG1,κ, BD Pharmingen) for 50 minutes at 4°C, respectively. After Fixation and Permeabilization, cells were incubated with CD68-Alexa Fluro 647 (clone ED1, IgG1, BIO-RAD) and Alexa Fluro 647 Isotype Control (clone ED1, IgG1, BIO-RAD) for 50 minutes at 4°C, respectively. Flow cytometric analysis was performed by using flow cytometry (BECKMAN COULTER CYTOFLEX S, USA, and BD Verse, USA).

Cytokine assessment. Supernatants of BALF were used to quantitatively measure 27 rat Cytokines/Chemokines by using Magnetic Bead Panel (MERK, RECYMAG65K27PMX).

WB. Lung tissue was lysed by using RIPA buffer (Thermo Scientific). Equal amounts of proteins were separated by 10% SDS-PAGE and transferred to PVDF membrane(BIO-RAD). The membrane was blocked with 5% BSA (Sigma) for 90 minutes at room temperature, and incubated with primary antibody for 14 hours at 4°C. The primary antibody included anti-PPAR- γ (Abcam), anti-TGFβ1 (Proteintech), anti-phosphor-Smad3 (Cell Signaling Technology), anti-Smad3 (Cell Signaling Technology), anti-phosphor-Stat6 (Cell Signaling Technology), anti-Stat6 (Santa Cruz), anti-phosphor-Stat3 (Cell Signaling Technology), anti-Stat3 (Santa Cruz), anti-β-tubulin(Proteintech), anti-NFκB P65 (Abcam), anti-IκBα (Abcam), anti-IL1β (Abcam), anti-phosphor-NFκB P65 (Cell Signaling Technology), anti-phosphor-IκBα(Cell Signaling Technology), and anti-GAPDH (Abcam). And then, membranes were incubated with anti-rabbit/mouse IgG (H + L) (Abcam) for 1 hour at room temperature. Chemiluminescence signal was performed by using Amersham Imager 680(GE Healthcare Life Science, USA).

Histological staining. The lavaged lung (left lung) was then inflated with 4% formaldehyde, and maintained at a pressure of 25cmH₂O to keep for histological assessment. Sections (5µm) were

measured with hematoxylin and eosin (H&E) staining to investigate lung morphometric change.

Immunofluorescent staining. Formaldehyde-fixed lung sections (5µm) were dewaxed in xylene and rehydrated in ethanol/water. After antigen repair solution, 0.05% Triton×100 permeabilization, and blocked with 10% goat serum, the sections were incubated with primary antibody, including a rabbit anti-PPAR-γ (Abcam), a rabbit anti-phosphor-Stat6 (Cell Signaling Technology), a rabbit anti-TGFβ1 (Proteintech), a rabbit anti-phosphor-Smad3 (Cell Signaling Technology), a rabbit anti-phosphor-NFκB P65 (Cell Signaling Technology), and a rabbit/mouse anti-CD68 (Abcam) at 4 °C overnight respectively. Sections were washed three times, and incubated with Alexafluor 488-conjugated goat anti-rabbit IgG (Abcam), Alexafluor 647-conjugated donkey anti-mouse IgG (Abcam), and CoralLite 594-conjugated goat anti-rabbit IgG (Proteintech) at 37°C for 1 hour, and then labeled with DAPI for 5minutes. Immunofluorescence was performed by a confocal microscope (Carl Zeiss, Oberkochen, Germany).

Statistical analysis. Statistical analyses were performed by IBM SPSS 22.0, and data were expressed as mean ± SD. Two group comparisons were accomplished by an unpaired t-test. More than two groups comparisons were analysed using one-way ANOVA test. Mann-Whitney U-tests were used to compare relative mRNA expression and CD206 MFI between experimental groups. $p \leq 0.05$ was considered significant.

Results

Determination of particle size distributions and gas concentrations in the exposure chamber. To measure the particle size distributions in suspension and gas concentrations, we used Dust Trak aerosol detector (TSI, Shoreview, USA) and smoke Test340 portable gas analyzer (Testo, Lenzkrch, Germany) to evaluate the quality control parameters of the exposure system. The value of PM₁, PM_{2.5}, and PM₁₀ were 27.77 ± 8.66 mg/m³, 28.07 ± 8.84 mg/m³, and 28.23 ± 8.86 mg/m³ in the BMF exposure room, respectively (Supplementary Table 2). The CO concentration was maintained at a low level of 55.16 ± 13.77 ppm, and NO and SO₂ weren't detected.

BMF induced Lung morphological changes and AMs infiltration. In order to investigate whether air pollution matter exposure causes emphysema in our exposed rat model, we did hematoxylin and eosin (H&E) staining to examine lung morphometric character. Alveolar enlargement was calculated as the mean linear intercept (MLI), and the bronchial wall thickness was quantified by wall thickness = (total bronchial area-lumen area)/total bronchial area. Our data showed that BMF exposure induced emphysematous changes and airway remodeling (Fig. 1a-d). Long-term BMF exposure damaged the lung parenchyma and airway wall, which led to alveolar enlargement and distal airway remodeling. Histological analysis demonstrated that the airway wall thickness increased ($p \leq 0.01$), and the mean linear intercept decreased dramatically ($p \leq 0.01$) at 6 months, whereas, there was no change at 1 month compared to controls ($p = 0.366$ and 0.557). Total BALF cells in BMF exposure groups were increased compared with the control groups after 4 days, 1 month, and 6 months BMF exposure (Fig. 1e, $p = 0.013$, 0.001 , and 0.003 , respectively). AMs were labeled with pan macrophage surface marker CD68, and defined as CD68 +

subpopulation with the purity displayed as a percentage of parent population gated on FSC-A/SSC-A. The numbers of infiltrated AMs were more than CON groups after 4 days, 1 month, and 6 months BMF exposure (Fig. 1f, $p = 0.01$, 0.04 , and 0.003 , respectively), and reached a peak at 6 months of BMF exposure.

BMF exposure induced BALF cytokine expression. In order to investigate how BMF exposure influences pulmonary inflammation, which may affect M1/M2 phenotype, 27 cytokines/chemokines multiplex tests were performed (Fig. 2). We measured G-CSF, Eotaxin, GM-CSF, IL-1 α , Leptin, MIP-1 α , IL4, IL1 β , IL2, IL6, EGF, IL13, IL10, IL12p70, IFN γ , IL5, IL17A, IL18, MCP-1, IP10, GRO/KC/CINC-1, VEGF, Fractalkine, LIX, MIP-2, TNF α , and RANTES protein levels by using Rat Cytokine/Chemokine Magnetic Bead Panel. It showed that IL1 α , IL1 β , IL12p70, LIX, EGF as well as VEGF increased significantly after 4 days of BMF exposure ($p = 0.018$, 0.008 , 0.043 , 0.001 , 0.001 , and 0.007 , respectively). IL1 β , TNF α , and LIX were higher than the control groups at 1 month ($p = 0.038$, 0.031 , and 0.021 , respectively). Compared to the control groups, there was no cytokine change after 6 months of BMF exposure except for VEGF ($p = 0.014$). The result indicated that high levels of inflammatory cytokines were induced in the early stage of BMF exposure. Interestingly, cytokine analysis also showed that IL4 in BALF increased significantly compared to the control group after 4 days of BMF exposure ($p = 0.002$), and restored to near controls during the subsequent time ($p = 0.124$ and 0.118).

Phenotypic characterization of AMs polarization induced by BMF exposure. In order to investigate gene expression of AMs when exposed to BMF, we also used quantitative PCR to determine the mRNA expression for a few key genes (Fig. 3). The result showed that iNOS and IL1 β significantly ascended at 4 days of BMF exposure (Fig. 3a, $p = 0.005$ and 0.001), and descended to near normal levels during the subsequent time. TNF α moderately elevated in 1 month BMF exposure (Fig. 3b, $p = 0.028$), and declined in 6 months BMF exposure (Fig. 3c, $p = 0.374$), consistent with BALF cytokines expression. Whereas, TLR2 and TLR4 had no change during the whole exposure course, consistent with previous studies^{30,31}. The level of EGF mRNA was upregulated in AMs of rats exposed to 4 days BMF (Fig. 3a, $p < 0.01$), which was consistent with the level of EGF protein expression in BALF.

To further investigate the effect of BMF exposure on the dynamic phenotype change of AMs in rats, in addition to the gene expression, we assessed the CD206 (M2 marker) and CD86 expression (M1 marker) in AMs (Fig. 3). The result showed that CD206 MFI decreased at 4 days of BMF exposure (Fig. 3d,e, $p < 0.01$), and increased to near controls following 1 month exposure ($p = 0.207$), and was significantly higher than the control group following 6 months exposure ($p = 0.035$). Conversely, CD86 MFI had no change in AMs during the whole exposure of biomass fuel smoke (Fig. 3f,g, $p = 0.730$, 0.831 , and 0.995 , respectively). The result indicated that BMF exposure reduced the anti-inflammatory marker expression in AMs at the beginning, and the anti-inflammatory marker expression was increasing with the accumulation of exposure time.

BMF exposure triggered signaling pathways of macrophage polarization and activation. To study which signaling pathways involved in AMs polarization and activation under the BMF exposure, especially those

involved in M2 polarization to attenuate the inflammatory response and promote tissue remodeling^{13,32}, such as Stat6, Stat3, PPAR γ , and TGF β 1. We used quantitative PCR, western blot, and immunofluorescence to determine the mRNA and protein level of Stat6, Stat3, PPAR γ , and TGF β 1 in BMF exposed rats. It showed that Stat6 mRNA expression in AMs increased significantly after 4 days of BMF exposure (Fig.S1a, $p < 0.01$), and descended to near controls during the subsequent time (Fig.S1b,c, $p = 0.149$ and 0.661). The level of p-Stat6 increased after 4 days of BMF exposure (Fig. 4a,b, $p < 0.01$). Stat3 mRNA expression in AMs had no change compared to control groups after 4 days, 1 month, and 6 months of BMF exposure (Fig.S1a,b,c, $p = 0.112$, 0.209 and 0.832). In contrast, the level of p-Stat3 level elevated after 4 days of BMF exposure (Fig. 4a,b, $p = 0.003$), and then declined to near control group after 1 month and 6 months exposure (Fig. 4a,b, $p = 0.898$ and 0.484). PPAR γ mRNA expression in AMs elevated at 4 days of BMF exposure (Fig.S1a, $p < 0.01$), and declined to a normal level during the subsequent course (Fig.S1b,c, $p = 0.66$ and 0.543). There was no change of PPAR γ protein in lung tissue between controls and exposure groups after 1 month and 6 months of BMF exposure (Fig. 4a,b, $p = 0.934$ and 0.572). But, PPAR γ protein significantly increased at 4 days of BMF exposure (Fig. 4a,b, $p = 0.005$), consistent with PPAR γ mRNA expression in AMs.

On the other hand, TGF β 1 and p-Smad3 protein levels in lung tissue didn't increase until 6 months of BMF exposure (Fig. 5a-c, $p = 0.017$ and 0.017). Additionally, AMs expressing p-STAT6, PPAR γ , TGF β 1, and p-Smad3 were double examined by Immunofluorescence staining (Fig. 4c-d, 5d-e). It showed PPAR γ , p-STAT6, TGF β 1, and p-Smad3 protein were induced in CD68 positive cells (AMs) after 4 days and 6 months of BMF exposure, consistent with PPAR γ , p-STAT6, TGF β 1, and p-Smad3 protein level in lung tissue.

PPAR γ primed BMDMs exposed to PM into alternative macrophages. To study whether PPAR γ reversed M1 phenotype induced by biomass ambient particulate matter, and drove the macrophages into M2 phenotype, we cultured bone marrow derived macrophages(BMDMs), and stimulated them with PM extracted in our lab, and used PPAR γ agonist and PPAR γ KO lentivirus as the intervention in vitro. 30 μ g/ml PM was selected as an intervention concentration (Fig.S2a-c). The effect of PPAR γ on inflammatory factors was determined via quantitative PCR, western blot, and immunofluorescence. 30 μ g/ml PM induced BMDMs to secrete iNOS, IL1 β , TNF α , and TLR2 (Fig. 6a, $p < 0.01$ for all genes), and BMDMs showed a pro-inflammatory phenotype. Transcriptional factor PPAR γ inhibited pro-inflammatory genes (Fig. 6a, $p < 0.01$ for all genes). 30 μ g/ml PM triggered phosphorylation of IKB α from 6 hours to 12 hours, and triggered p-P65 began to rise after 6 hours (Fig.S3a,b). PPAR γ overexpression significantly inhibited phosphorylation of P65 and IKB α (Fig. 6b, $p < 0.01$ for all comparisons). PPAR γ overexpression also repressed upregulation of IL1 β protein level induced by PM (Fig. 6b). PPAR γ KO lentivirus increased the level of p-P65 and p-IKB α (Fig. 6c, $p < 0.01$ for all comparisons). Immunofluorescence staining showed that PPAR γ KO lentivirus promoted p-P65 nucleus translocation, while PPAR γ overexpression inhibited p-P65 nucleus translocation induced by PM (Fig. 6d).

Our data also showed that PPAR γ in BMDMs was upregulated by stimulation with IL4, and promoted the expression of M2 markers. IL4 stimulated PPAR γ and p-STAT6 expression (Fig. 7a,b, $p < 0.01$). PPAR γ

overexpression increased the level of p-STAT6 (Fig. 7a,b, $p < 0.01$), while PPAR γ KO lentivirus attenuated phosphorylation of STAT6 ($p < 0.01$). In addition, PPAR γ overexpression upregulated CD206 expression (Fig. 7c, $p = 0.026$). PPAR γ KO lentivirus downregulated CD206 expression (Fig. 7c, $p = 0.041$).

TGF β 1 promoted CD206 expression in BMDMs. 30 μ g/ml PM decreased CD206 expression (Fig. 8, $p < 0.01$), TGF β 1 attenuated the downregulation of CD206 induced by PM (Fig. 8, $p < 0.01$), but had no effect on BMDMs without stimulation of PM ($p = 0.522$).

Discussion

Indoor air pollution induced by biomass ambient particulate matter is strongly link to incidence and hospitalization rates of COPD. Our previous study showed that biomass ambient particulate matter retention in lung tissue induced pulmonary inflammation, airway remodeling, and alveolar cavity enlargement. The chronic BMF exposure model serves as a useful model to analyze how indoor air pollution promotes the progress of emphysema in lung. In this work, we demonstrated that the emergence of pro-inflammatory macrophage eventually conversed into anti-inflammatory macrophage associated with BMF induced inflammation, which revealed that biomass ambient particulate matter initiated this plasticity in AMs. In addition, functional conversion of AMs was regulated by signal pathways. We also found the dynamic change of some regulated molecules, which drove AMs into an anti-inflammatory phenotype.

Airway inflammation, airway remodeling, and alveolar cavity enlargement were observed in our BMF exposure model. Our data showed that the early stage of BMF exposure models was characterized as airway inflammation, and the later stage was characterized by airway remodeling and alveolar cavity enlargement, which was in agreement with previous observation in COPD models³³. Our previous study also showed that PEF and FEV(20)/FVC in 7 months exposure group were significantly lower than the control group, indicating that BMF induced dysfunction of lung function³³. CD68 is a specific macrophage marker in rats, mice, and humans, and F4/80 is expressed in mature macrophages in mice. CD11b is expressed in both rats and mice, but is low expressed in AMs³⁴. In this study, we used CD68 as a marker of AMs. Our data showed that BMF exposure induced macrophages infiltration in BALF, and the number of AMs increased with the accumulation of exposure time.

Surprisingly, molecular analyses revealed that the emerging pro-inflammatory macrophages shared a feature of anti-inflammatory phenotype, which was partially overlapping but also distinct, including the co-expression of mRNA encoding of IL1 β , iNOS, and PPAR γ . The result also indicated that AMs produced pro-inflammatory factors to damage lung tissue and subsequently skewed towards anti-inflammatory phenotype after short-term exposure. After 6 months of BMF exposure, the protein level of TGF β 1 increased, and airway and lung tissue were remodeled, which resulted in the COPD pathology. The whole course from pro-inflammatory phenotype to anti-inflammatory phenotype, and then to the chronic pathology, indicated an interaction between BMF exposure and the pulmonary immune system.

Inflammation happened in the early stage of BMF exposure. Camila Oliveira da Silva and co-workers observed a dynamic change of cytokine production in CS exposure mice. They found that TNF α and NO increased in five days CS exposure mice, and then greatly decreased in 14 days of CS exposure. Afterward, 30 days of CS exposure increased TGF β 1 production in the lung³⁵. We also found IL1 α , IL1 β , IL12p70, LIX as well as IL4 increased remarkably in BALF exposed to short-term biomass fuel smoke. IL1 α/β , which are mainly produced by activated monocytes and macrophages, enhance B cell proliferation and maturation, NK cytotoxicity, pro-inflammatory chemokine expression, as well as acute phase protein expression. IL12p70, produced by monocytes and macrophages, can further act on lymphocytes, and effectively promote Th1 response in COPD. LIX, which is a small cytokine of the CXC chemokine family, is produced by epithelial cells following stimulation with IL1 or TNF α , and promotes the chemotaxis of neutrophils. Pro-inflammatory cytokine dynamic change revealed that the most severe inflammatory injury is in the early stage of exposure models. Interestingly, Th2 cytokine IL4 also increased simultaneously, which was able to promote AMs towards anti-inflammatory phenotype. Then the pro-inflammatory cytokines gradually decreased during the subsequent exposure course. The increase of IL4 and the recovery of inflammation didn't match in time in COPD models, which was in conformity with the previous study in acute exacerbation of chronic obstructive pulmonary disease³⁶. Previous studies have reported that M2 secreted EGF and VEGF to promote tissue repair^{17,37}. The data suggested that EGF and VEGF upregulated under the action of Th2 cytokine in the early stage of BMF exposure. Renat Shaykhiev and co-workers provided transcriptome-base evidence that smoking induced reprogramming towards M2 polarized macrophage in COPD patients for the first time, suggesting that AMs were likely involved in COPD pathogenesis in a non-inflammatory manner³⁸. However, the clinical study could not track the change of immune cells on the time axis. Our study provided the dynamic phenotype and functional changes of AMs during BMF exposure.

Macrophage polarization is controlled by signal pathway molecules. Which signal pathways regulate macrophages have yet to be identified in the COPD model. In the present study, anti-inflammatory phenotype took the most time of BMF exposure period, and overlapped with pro-inflammatory phenotype at 4 days of BMF exposure. We focused on signal pathways of anti-inflammatory phenotype in the BMF exposure model more. IL4 skews macrophages toward the M2 phenotype, and activates Stat6 and Stat3. Stat6 modulates many genes associated with the M2 phenotype, including CD206, arginase 1, and resistin like³⁹. The present study found phosphorylation of Stat6 and Stat3 increased after 4 days of BMF exposure compared to controls, consistent with upregulation of IL4. It supported that Stat6 and Stat3 signals participated in activation of M2 in the early stage.

PPAR γ is one of the important transcription factors of M2, and primes monocytes into alternative activated macrophages^{20,40}. The dynamic change of PPAR γ has not been reported in previous studies. Our data showed that PPAR γ elevated during the most severe inflammation periods, and then returned to the normal level as inflammation disappeared. We further demonstrated that PPAR γ was upregulated in BMDMs by IL4 in vitro. The Th2 cytokine IL4 was required for the development of M2, and IL4 mediated signals stimulated PPAR γ expression^{20,41,42}. In addition, our study showed that PPAR γ significantly

inhibited activation and nucleus translocation of NF κ B p65 via repression of I κ B α phosphorylation, and upregulated M2 markers CD206 and p-STAT6 expression, suggesting PPAR γ reversed M1 phenotype induced by PM, and drove the macrophages into M2 phenotype. PPAR γ rapidly drove the transformation of AMs into anti-inflammatory phenotypes, and played a protective role in the short-term of BMF exposure in the study. There are some drugs in the PPAR γ pathway, which provide a potential target for intervention in COPD. Simon Lea and co-workers found that rosiglitazone inhibited cigarette smoke-induced pulmonary inflammation⁴³. Rosiglitazone was able to reduce exacerbations through attenuating pulmonary inflammation and decreasing bacterial burden⁴⁴. As a result, PPAR γ may be an effective approach for the treatment of COPD.

TGF β 1 is another important regulatory molecule of M2. TGF β 1 is a critical cytokine for the development and maturation of AMs. TGF β 1 also repressed macrophage-derived inflammatory gene^{45,46}. M2 participates in the development of fibrosis and contributes to disease pathogenesis. M2-derived TGF β 1 promoted tissue remodeling and wound repair by blocking the extracellular matrix's degradation and eliciting synthesis of interstitial fibrillar collagens¹³. Besides, it's confirmed that airway remodeling via the TGF β 1 pathway leads to the thickening of the small airway wall. TGF β 1 in AMs may be involved in the mechanism of airway remodeling. Our study showed that AMs maintained an anti-inflammatory phenotype characterized by elevated CD206 and TGF β 1 markers without stimulation of Th2 cytokines at 6 months of BMF exposure. We also found that TGF β 1 promoted CD206 expression in BMDMs in vitro. Chronic BMF exposure induced TGF β 1 production, and activated the downstream signal Smad3 involved in tissue remodeling. Activation of TGF β 1 in the late stage of BMF exposure, indicated the role of TGF β 1 in regulating M2 polarization and participating in lung tissue remodeling in BMF models.

Conclusion

Our study provided the dynamic phenotype and functional change of AMs during BMF exposure, suggesting AMs play a pro-inflammatory role in the early stage and an anti-inflammatory role associated with promoting tissue remodeling in the latter stage of COPD. Further identification of a few signal pathway molecules involved in AMs polarization under BMF exposure, may provide potential targets for the COPD treatment.

Abbreviations

AM: alveolar macrophage

COPD: chronic obstructive pulmonary disease

BMF: biomass fuel

BMDM: bone marrow derived macrophage

MAPKs: mitogen-activated protein kinases

α -SMA: α -smooth muscle actin

Declarations

Ethics approval and consent to participate

Guangzhou Medical University Animal Research Ethics Board approved the study(GY2018-088). The experimental protocol and animal care was in compliance with the guiding principles for the care and use of laboratory animals recommended by the Chinese Association for Laboratory Animal Science Policy.

Consent for publication

Not applicable.

Competing interest

The authors declare that no conflict of interest exists.

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Author contributions

Shenlin Wang designed study, performed experiments, analyzed data, and wrote the manuscript. Yuhua Chen performed experiments and analyzed data. Fan Wu analyzed data. Wei Hong, Bing Li, and Yumin Zhou created the air pollution exposure system. Pixin Ran conceived and designed experiments, and assisted with manuscript preparation. All authors read and approved the final manuscript.

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Figures

Figure 1

Lung morphological changed, and AMs infiltrated in BALF following BMF exposure. Bars are equal to 50 μm . a,c show small airway wall with HE staining and statistical analysis of small airway wall thickness. b,d show lung tissue with HE staining and statistical analysis of mean linear intercept(MLI). e Comparison of the total number of BALF cells between BMF and CON groups. f Comparison of AMs in BALF between BMF and CON groups. Data in c,d,e, and f represent the mean $\pm$ SD of a minimum number of 6 rats per group. *p<0.05, ** p<0.01, significantly different from clean air groups.

Figure 2

BMF induced cytokine expression in BALF. Rat 27 cytokine analysis showed L1α, IL1β, IL12p70, LIX, TNFα, IL4, EGF, and VEGF altered in BALF significantly. Data represent the mean ±SD of a minimum number of 6 rats per group. *p<0.05, ** p<0.01, significantly different from clean air groups.

Figure 3

BMF altered the expression of genes and surface markers in alveolar macrophages. a iNOS, IL1 β , and EGF mRNA expression upregulated in AMs exposed to BMF 4 days. b TNF α mRNA expression in AMs had changed after 1 month of BMF exposure. c iNOS, IL1 β , TNF α , TLR2, TLR4, and EGF mRNA expression had no changed after 6 months of exposure. d,e Comparison of CD206 expression in AMs between BMF and CON groups. f,g Comparison of CD86 expression in AMs between BMF and CON groups. Data represent the mean $\pm$ SD of a minimum number of 6 rats per group. * $p < 0.05$, ** $p < 0.01$, significantly different from clean air groups.

Figure 4

BMF exposure triggered Stat6, Stat3, and PPAR γ activation. a,b Comparison of p-Stat6, p-Stat3, and PPAR γ protein expression in lung tissue between groups. c Time-dependent activation of p-Stat6 was examined by double Immunofluorescence staining of p-Stat6 (green) and CD68 (red). d Time-dependent activation of PPAR γ was examined by double Immunofluorescence staining of PPAR γ (green) and CD68 (red). Bars are equal to 20 μ m. The value in a and b represent the mean $\pm$ SD of a minimum number of 6 rats per group. * $p < 0.05$, ** $p < 0.01$, significantly different from CON groups.

Figure 5

BMF exposure triggered TGFβ1 pathway activation. a,b Western blotting showed comparison of TGFβ1 protein expression in lung tissue between groups. a,c Western blotting showed protein level of p-Smad3 and Smad3 in lung tissue. d TGFβ1 in AMs was examined by double Immunofluorescence staining of TGFβ1 (green) and CD68 (red). e p-Smad3 in AMs was also examined by double Immunofluorescence staining of p-Smad3 (green) and CD68 (red). Bars are equal to 20μm. The value in b and c represent the

mean $\pm$ SD of a minimum number of 6 rats per group. * p <0.05, ** p <0.01, significantly different from CON groups.

Figure 6

PPAR γ reversed M1 phenotype induced by biomass ambient particulate matter. a Comparison of iNOS, IL1 β , TNF α , and TLR2 mRNA expression in BMDMs between groups after 24 hours. b,c Western blotting showed comparison of p-IK β expression in BMDMs between groups after 6 hours, and comparison of p-P65 and IL1 β expression after 48 hours. d Effect of PPAR γ on nucleus translocation of p-P65 in BMDMs. Nucleus translocation of p-P65 in BMDMs between groups after 48 hours was examined by immunofluorescence staining of p-P65(red). Bars are equal to 10 μ m. The value in a,b and c represent the mean $\pm$ SD of a minimum number of 6 independent experiments. * p <0.05, ** p <0.01, significantly different from control groups.

Figure 7

PPAR γ primed BMDMs into M2 phenotype. a,b Western blotting showed comparison of PPAR γ and p-STAT6 expression in BMDMs between groups. c Comparison of CD206 MFI in BMDMs between groups. The value in b and c represent the mean $\pm$ SD of 6 independent experiments. * p <0.05, ** p <0.01, significantly different from control groups.

Figure 8

TGFβ1 promoted CD206 expression in BMDMs. Comparison of CD206 MFI in BMDMs between groups. The value in b represents mean $\pm$ SD of 6 independent experiments. * $p < 0.05$, ** $p < 0.01$, significantly different from control groups.

Figure 9

Schematic representation of AMs activation and polarization triggered by BMF exposure. Following 4 days of BMF exposure, pro-inflammatory factors increased, such as iNOS, IL1α, IL1β, and IL12p70, while

alveolar macrophages surface marker CD206 decreased remarkably. Interestingly, the emerging pro-inflammatory macrophages shared a signature of activated anti-inflammatory, which was partially overlapping. Consistent with IL4 upregulated in BALF, M2 transcriptional signal molecules in AMs elevated simultaneously, such as PPAR γ and p-Stat6. After 6 months of exposure, CD206, TGF β 1, and p-Smad3 in AMs were significantly higher than the control group, and emphysema and airway remodeling happened.

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